Exhibit A

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Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen

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Direct gene transfer by intramuscular injection of plasmid DNA encoding an antigenic protein may be used for the purpose of immunization. DNA-based immunization may be of value for basic immunological research and vaccine development. Several factors influence the uptake and expression of plasmid DNA in skeletal muscle, which in turn influence the immune response to the expressed protein. Physical barriers and other factors may impede diffusion of the DNA within the muscle tissue or its entry into the muscle fibres. Although the efficiency of gene transfer in normal mouse muscle is low (< 100 fibres per injection site), a humoral response to the hepatitis B surface antigen (HBsAg) is obtained after expression of a transferred gene. Direct gene transfer is ten times more efficient in regenerating than in normal mouse muscle. DNA-based immunization in such regenerating muscles results in an earlier and stronger humoral response to HBsAg than is seen in normal mature muscle. A needleless jet injection system (Biojector®) is able to deliver DNA into normal muscle in rats and rabbits such that a substantial immune response is obtained.

Keywords: Hepatitis B; skeletal muscle; plasmid expression

The term DNA-based immunization currently refers to the induction of an immune response to a protein antigen expressed *in vivo* subsequent to the introduction of purified plasmid DNA encoding the polypeptide sequence. The resulting *in situ* production of the protein can involve biosynthetic processing and post-translational modifications. This method of immunization differs from the use of classical vaccines, which are composed of the antigen itself, either in the form of the whole pathogen, a killed or attenuated virus or bacteria, or a subunit component of it (e.g. purified or recombinant proteins or synthetic peptides). If introduction of plasmid DNA is both efficient and safe, nucleic acid-based immunization may one day be an attractive alternative to classical vaccines, particularly in the preparation of multi-component vaccines.

The direct *in vivo* transfer of genes can be accomplished in many tissue types and by several different means[1]. Since routine vaccination is almost always applied to large populations, direct (*in vivo*) gene transfer would be the method of choice for prophylactic immunization purposes. Although indirect gene transfer, which involves re-implantation of cells removed from an individual and transfected *ex vivo*, might be considered for certain forms of immunotherapy, it is too cumbersome and expensive to consider unless direct gene transfer methods fail.

Direct gene transfer may be carried out using either viral vectors or recombinant plasmid DNA carrying cloned genes to be expressed *in situ*. The use of pure plasmid DNA offers many potential advantages over the viral vectors for the purpose of immunization. These include greater ease and speed of production, easier quality control, non-integration of the DNA and lack of immunogenicity of the vector itself[2,3]. Other advantages which are particularly important for use in developing areas of the world include the ease and lower cost of production, as well as the heat stability of DNA (as opposed to most vaccine preparations) which could allow DNA to be transported and stored in a lyophilized form. It has been demonstrated in several studies that plasmid DNA is taken up and expressed more efficiently in skeletal and cardiac muscle than in other tissues, although further research may well reveal additional tissues which can be adequately transfected. Although the basis for the higher efficiency of muscle tissue to take up DNA is not precisely known, it has been suggested that this is due to the presence of t-tubules, which are a unique feature of striated muscle.

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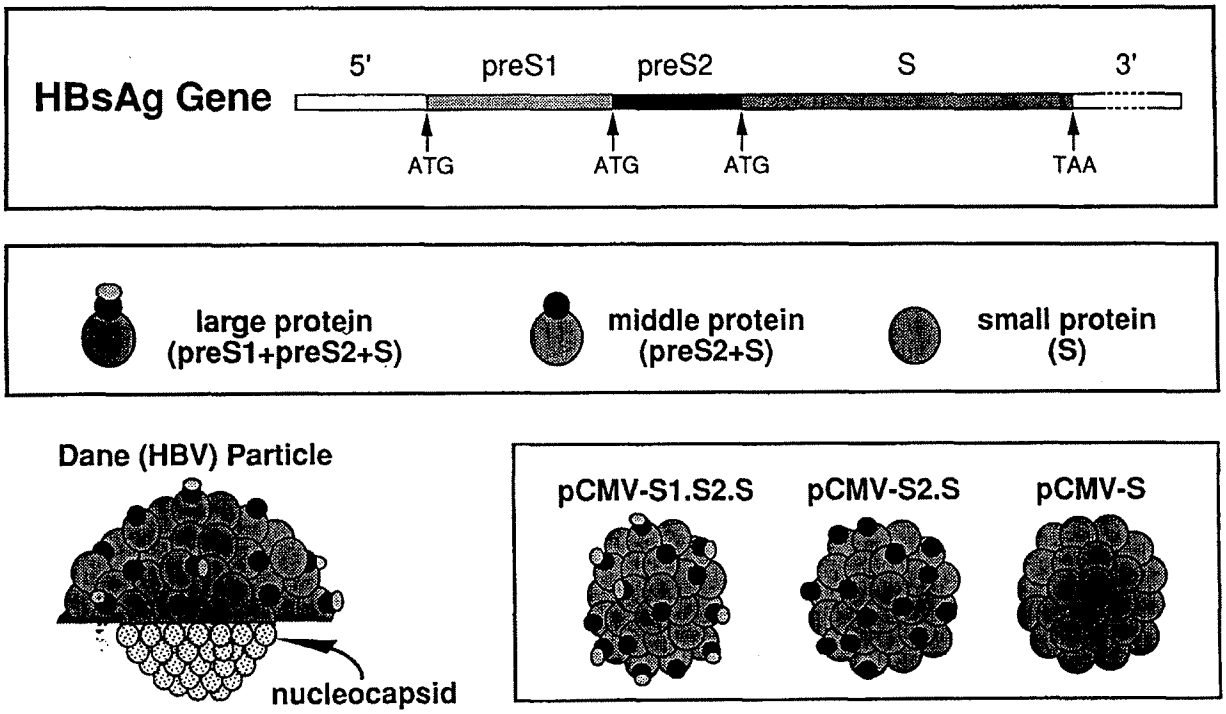

Figure 1 HBsAg is encoded by a single gene which is divided into S, pre-S2 and pre-S1 regions by three ATG start codons. Three polypeptides are thus encoded by this gene: the small (S), middle (pre-S2+S) and large (pre-S1+ pre-S2+S) proteins. A messenger RNA transcript containing all three ATG codons has the ability to produce all three forms of the protein since internal initiation can take place. The envelope of infectious viral particles (Dane particles) contains all three forms of HBsAg, but with a predominance of the small (S) protein. We have constructed three DNA expression vectors based on the plasmid pcDNA3 (Invitrogen Corp.) which can encode the synthesis of empty viral envelope particles containing only the small (S) protein[18] or particles containing mixtures of the small and middle (S2.S) or large (S1.S2.S) proteins (unpublished results). Particles of the type encoded by the vector pCMV-S1.S2.S are found in the serum of chronically infected individuals. The small S protein can be produced in yeast[23] as a recombinant protein for the purpose of vaccination

Skeletal muscle is an appropriate tissue for gene transfer for the purposes of immunization since it is both readily accessible and is currently a preferred site for administering vaccines. Although gene transfer into skeletal muscle can be carried out directly using intramuscular injection of adenovirus[3-5], retrovirus[3,6,7] or pure plasmid DNA[3,8-16], the highest level of transfer in mature skeletal muscle is obtained with plasmid DNA, even though at best only a small number of fibres (< 100) are transfected per injection site[3]. It is therefore possible to benefit from the clear practical advantages of plasmid DNA over the use of viral vectors.

At present, the biggest improvement in the efficacy of gene transfer into skeletal muscle, using either pure DNA or viral vectors, has involved their introduction into a regenerating muscle mass. The regenerating tissue probably allows greater diffusion of injected substances compared with normal adult muscle, and some properties of the immature regenerating muscle fibres may render them more susceptible to taking up DNA. In a direct comparison, we have found that adenovirus and plasmid DNA give equivalent results in regenerating muscle, and that both are superior to the use of retrovirus[3]. Muscle regeneration can be easily and reproducibly induced in adult muscle tissue by the use of necrotizing agents such as snake toxins[17] or local anaesthetics[6]. Although these treatments may reasonably be considered unfeasible for widespread human use, they nonetheless provide useful experimental models in animals, and could potentially be employed in certain human therapeutic protocols where increased DNA uptake is an important factor. Finally, the greater DNA transfer obtained in regenerating muscle suggests that other more acceptable formulations could be found which would improve the efficacy of DNA transfer in muscle tissue.

We have undertaken a study of the factors which determine the efficiency of direct gene transfer using plasmid DNA in muscle tissue[3,14,15]. This has been studied particularly with respect to the influence of DNA transfer methods on the immune response obtained after DNA-based immunization[18]. We have studied three models or methods by which plasmid DNA expression vectors can be introduced into mammalian skeletal muscle for the purposes of immunization. Furthermore, we have used a model of DNA-based immunization against the surface antigen of the hepatitis B virus (HBsAg) and have evaluated the humoral response to this antigen with these gene transfer methods.

HBsAg is encoded by a single gene with a large open reading frame. It contains three in-frame ATG start codons which divide the gene into three regions known as S, pre-S2 and pre-S1 (see *Figure 1*). The three different sized polypeptides which can be produced are known as the small (S), middle (pre-S2+S) and large (pre-S1+pre-S2+S) proteins. The envelope of the infectious (Dane) particle of the hepatitis B virus (HBV) contains all three forms, but with a predominance of small protein and variable amounts of middle and large proteins. The serum of infected individuals also contains large numbers of empty viral envelopes composed solely or predominantly

of small envelope protein and sometimes small amounts of the middle and trace amounts of the large protein[19]. The first vaccine against HBV to be used in humans involved injection of such particles, which had been purified from the plasma of chronic carriers[20]. This plasma-derived protein vaccine was effective, but was not appropriate for mass immunization owing to the limited supply of chronically infected human plasma[21,22], Recombinant protein vaccines composed of empty particles containing either only the small S protein[23] or the middle and small proteins[24] are currently used. We have produced plasmid DNA expression vectors for HBsAg that are designed for expression in muscle, and which will result in the synthesis of particles containing either the small protein only or mixtures of the small, middle and large proteins (*Figure 1*).

MATERIALS AND METHODS

Plasmid DNA expression vectors

For reporter gene expression, recombinant plasmid DNAs contained either the firefly *Photinus pyralis* luciferase gene (*luc*) or the *Escherichia coli* β-galactosidase gene (*lacZ*) driven by the immediate–early promoter of human cytomegalovirus (CMV)[25]. These two vectors have been described previously: pCMV-luc[26], pCMV-lacZ[27].

For expression of HBsAg, the surface antigen coding sequences and the 5′ and 3′ non-coding sequences including the polyadenylation signal were cloned into an expression vector driven by the CMV promoter. The vector used in all the experiments presented, designated here as pCMV-S, has been described previously[18].

The plasmid DNA used in all experiments was prepared according to the manufacturer's directions using Qiagen DNA purification columns (Diagen, Hilden, Germany). Following isopropanol precipitation, the DNA was redissolved in 10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, the solution was adjusted to 0.1 M NaCl and the DNA was precipitated with 2.5 volumes of ethanol[28]. After washing the pellet in 70% ethanol, the DNA was redissolved in sterile, endotoxin-free Dulbecco's phosphate-buffered saline (PBS; Sigma) at a final concentration of 1–2 mg ml^{-1}, aliquoted and stored at −20°C until required for intramuscular injection.

In vivo gene transfer

Intramuscular injection of DNA expression vectors into normal and regenerating (see below) muscle of mice was performed as previously described[3,18]. In brief, male C57BL/6J mice (6–8 weeks old) received bilateral injections in the tibialis anterior (TA) muscles of DNA in a volume of 50 μl PBS. Each muscle was injected with 10 μg pCMV-luc (at 0.2 μg DNA per μl) or 50 μg pCMV-lacZ or pCMV-S (at 1 μg DNA per μl). All intramuscular injections into the mouse TA were through the skin using a 27 gauge needle fitted with a collar of polyethylene tubing which limited penetration to 2 mm.

Rats (male Wistar, 250 g) received intramuscular injections of DNA bilaterally into the gastrocnemius muscle using an ordinary syringe fitted with a 27 gauge needle inserted to a depth of 5 mm or using the Biojector® needleless jet injection system (Bioject Inc., Portland, OR) comprising a syringe with outlet orifice diameter of 0.1 mm. Each muscle was injected with 400 μl of DNA in PBS containing either 400 μg pCMV-lacZ or pCMV-luc (at 1 μg μl^{-1}) or 800 μg pCMV-S (at 2 μg μl^{-1}).

Rabbits (albino, 2–2.5 kg) were injected at four sites: bilaterally in both the gluteus maximus and quadriceps muscles. Each site received 500 μg pCMV-S (500 μl at 1 μg μl^{-1}) giving a total of 2 mg DNA per rabbit. Injections were carried out with either an ordinary syringe fitted with a 27 gauge needle and inserted to a depth of 1 cm or the Biojector® (as above).

All intramuscular injections in mice, rats and rabbits (of plasmid DNA or sucrose solution and cardiotoxin as discussed below) were carried out under general anaesthesia (sodium pentobarbital, 75 mg kg^{-1}, intraperitoneally).

Pretreatment with hypertonic sucrose

Normal mature mouse TA muscles were pretreated (15–30 min before gene transfer) by injection with 100 μl hypertonic sucrose (25% w/v in PBS).

Regeneration model

Regenerating muscle was produced by inducing a single cycle of myofibre degeneration and regeneration in the TA muscle by intramuscular injection of cardiotoxin (10 μM in PBS), which was purified from the venom of the *Naja nigricollis* snake (Latoxan, product reference L-8102; Rosans, France). This treatment has been shown to induce simultaneous degeneration of all myofibres, without affecting blood supply or innervation and without damaging the satellite cells, which are the stem cells required for regeneration[29]. Mouse TA muscles were each injected with 100 μl of the cardiotoxin solution, whereas 500 μl was used for the rat gastrocnemius muscles.

Assay of reporter gene expression

Luciferase activity was measured using the Promega luciferase assay system as described previously.[14]

Muscles receiving the *lacZ* reporter gene were removed 5 days after gene transfer, sectioned on a cryostat (12 μm) and stained for β-galactosidase as described by Dannenburg and Suga[30] and modified by Wolff *et al.*[12].

Assay of anti-HBsAg

At various times following gene transfer, blood was collected from anaesthetized mice by retrobulbar puncture using heparinized glass pipettes. The serum, which was recovered by centrifugation (10 min at 4000*g*), was stored at −20°C until used for assay of HBsAg or anti-HBsAg. The amounts of serum HBsAg and anti-HBsAg were measured using commercial ELISA methods (Diagnostics Pasteur or Abbott).

RESULTS

Sucrose pretreatment of muscle

We have previously shown that muscle injected with hypertonic sucrose 15–30 min before injection of plasmid DNA (pCMV-luc) does not show significantly higher levels of luciferase reporter gene activity than normal control muscle, but it does result in significantly less variability between animals.[14]

Table 1

State of mouse TA muscle	Reporter gene activity[a] Luciferase (1000 RLU s^{-1} mg^{-1})	β-galactosidase (number of fibres)
Normal mature	171 ± 83	36 ± 7
Regenerating (days)[b]		
1	17 ± 6	0
3	196 ± 125	39 ± 13
5	1771 ± 237	316 ± 49
7	1334 ± 431	
9	1135 ± 297	138 ± 28
11	1018 ± 448	

[a]Each value represents the mean ± s.e.m. of values obtained from six mouse TA muscles. RLU, relative light units. The activity of β-galactosidase is represented as the maximum number of stained fibres per cross-section of muscle

[b]The numbers represent the days following cardiotoxin-induced degeneration of TA muscle at which DNA was injected into the regenerating tissue

DNA-based immunization in regenerating muscle

Expression of the luciferase reporter gene (using pCMV-luc) in the mouse TA muscle was found to be tenfold greater in muscles which had been regenerating for 5–10 days (i.e. when maturing myofibres are present), than in normal (sucrose-pretreated) mature muscle. Although some gene expression was obtained after injection of DNA into muscles which had been regenerating for 3 days, when myotubes have already been formed, none was detected after injection at 1 day, when dividing satellite cells and mononucleated myoblasts predominate.

Similarly, approximately ten times more fibres stained for β-galactosidase in regenerating (5 days post-degeneration) than in normal muscles injected with pCMV-lacZ (*Table 1*). The staining was only detected within the muscle fibres themselves and not in mononucleated cells such as satellite cells or those found in the surrounding connective tissue.

DNA-based immunization to HBsAg resulted in a more rapid and greater production of antibodies in regenerating than in normal muscles in mice (*Figure 2*). For example, antibodies could be detected in immunized mice about 10 days earlier after DNA transfer into regenerating (as opposed to mature) muscle, and antibody levels after 1 month were about ten times greater. Similar results were obtained in rats, where antibody levels at 8 weeks were about 20 times greater following gene transfer into regenerating rather than mature muscle (results not shown). Nevertheless, even when the DNA was injected into normal muscle tissue, all animals eventually became seropositive and achieved antibody levels >10 mIU ml^{-1}, which is considered to be the minimum level capable of conferring protection in humans.

Biojector® jet injection system

Use of the Biojector® needleless injection jet system for DNA-based immunization (pCMV-S) in normal rabbit muscle with no pretreatment resulted in nearly fourfold greater antibody production at 8 weeks than with a normal syringe and needle (*Figure 4*). The Biojector® was also used successfully on rats to introduce DNA into normal and regenerating muscles for immunization against HBsAg (*Figure 3*). This apparatus could not be tested on mice since the force of the liquid jet stream is too powerful for such a small animal.

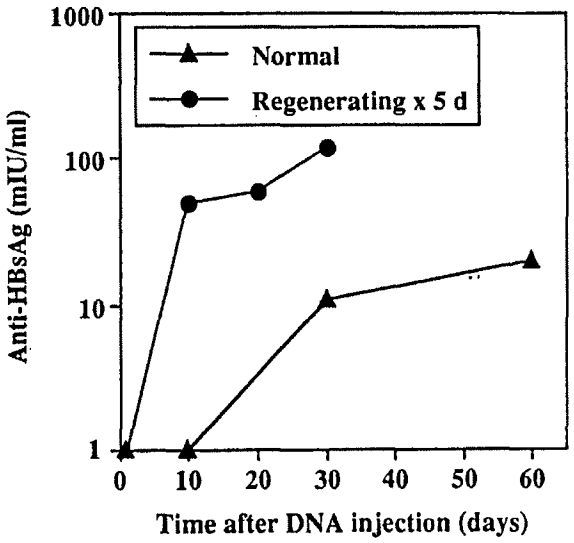

Figure 2 DNA-based immunization to HBsAg in mice which were injected bilaterally in the TA muscles with 50 μg pCMV-S in 50 μl PBS (100 μg DNA per mouse). The TA muscles had either been pretreated by injection with hypertonic sucrose (25% w/v in PBS) 15–30 min before DNA injection (normal group), or were undergoing regeneration following treatment with 10 μM cardiotoxin (100 μl) 5 days earlier (regenerating group). Antibody levels are expressed as mIU ml^{-1}, and each point represents the mean of values derived from 13 mice

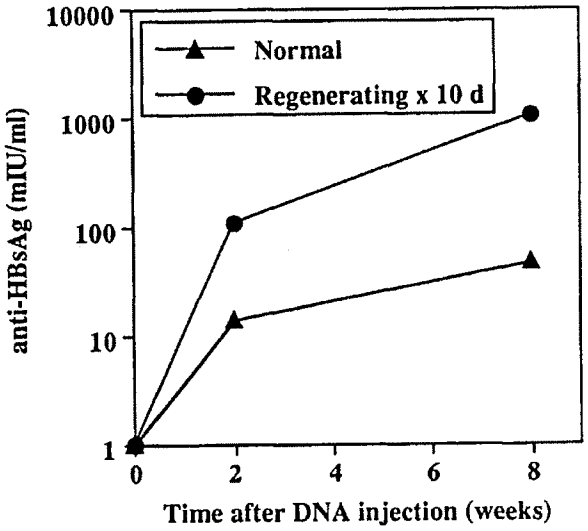

Figure 3 DNA-based immunization to HBsAg in rats which were injected bilaterally into normal (▲) or regenerating (●) (10 days post-degeneration) gastrocnemius muscles with 800 μg pCMV-S in 400 μl PBS (1.6 mg DNA per rat). Regeneration had been induced by intramuscular injection of 10 μM cardiotoxin (500 μl). All injections were carried out with the Biojector® needleless jet injection system. Antibody levels are expressed as mIU ml^{-1}, and each point represents the mean of values derived from four rats

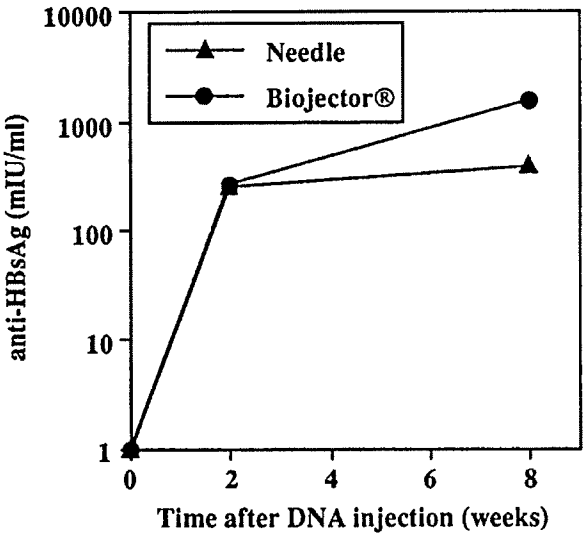

Figure 4 DNA-based immunizaton to HBsAg in rabbits which were injected at each of four sites with 500 μg pCMV-S in 500 μl PBS (2 mg DNA per rabbit). Injections were carried out either with a regular syringe and needle or with the Biojector" needleless jet injection system. Antibody levels are expressed as mIU ml^{-1}, and each point represents the mean of values derived from four rabbits

DISCUSSION

Barriers to direct gene transfer in muscle

Skeletal muscle is a highly differentiated tissue which requires special consideration with respect to gene transfer. An individual muscle cell, also known as a muscle fibre, is polynucleated and shaped like a tapered cylinder, and can exceed 1 m in length in humans. Each cell may contain hundreds of nuclei, which are located next to the cell membrane along the length of the fibre and are responsible for subserving a particular nuclear domain.

Direct gene transfer into normal mature muscle is not highly efficient regardless of the method used (i.e. plasmid DNA or viral vectors). At best, about 1–2% of the fibres in the TA muscle of the mouse (which contains approximately 3000 fibres) can be transfected with plasmid DNA. Far fewer numbers can successfully incorporate an adenoviral genome vector, and there is no uptake of retroviruses, which require dividing cells for stable integration of the genome[3]. Although the mechanism by which plasmid DNA enters the muscle cells is not known, it has been suggested that some feature particular to these tissues plays a role, and T-tubules have therefore been implicated[31]. T-tubules are invaginations of the plasma membrane which are responsible for conducting the depolarization potential into the interior of the cell so that the entire fibre can contract simultaneously, and are exclusive to striated muscle (cardiac and skeletal).

The relatively low efficiency of gene transfer in mature muscle may in large part be due to physical barriers which impede diffusion of the injected solution within the muscle tissue and entry of dissolved DNA into the muscle fibres. Muscle contains three levels of connective tissue sheaths. The endomysium surrounds individual muscle fibres and is continuous with the basal lamina of the cell, the mesomysium surrounds bundles or fascicles of fibres, and the epimysium encloses the entire muscle. The epimysium would not pose a barrier in direct intramuscular injection since it is perforated by the needle. In contrast, we have previously shown that the mesomysium does pose a barrier to the diffusion of substances injected intramuscularly. Examination of cross-sections of mouse muscle after injection with Indian ink showed that the ink was frequently restricted to interfascicular spaces over large regions of the muscle and did not surround individual muscle fibres[14].

Injection of a luciferase reporter gene construct into normal mouse muscle results in variable levels of expression, presumably because diffusion is poorer in some instances than others, depending on whether or not the injected DNA gains direct access to the intrafascicular compartment. This variability was ameliorated by pretreating the muscles with a hypertonic sucrose solution before DNA injection. However, the mean level of expression did not increase significantly, indicating that barriers at the level of the epimysium and/or cell membrane may also play a role. The role of diffusional barriers in limiting gene transfer in muscle has also been suggested for primate muscle, which has more extensive connective tissue than found in rodent skeletal muscle[11]. The role of diffusional barriers in limiting gene transfer is also indicated by the fact that, in the mouse diaphragm muscle, which is not extensively subdivided by connective tissue, the transfer of plasmid DNA is more efficient without pretreatment than in sucrose-treated hindlimb muscles[15].

Gene transfer in regenerating muscle

The multiple nuclei in skeletal muscle fibres are permanently postmitotic. A nearly total complement of muscle fibres is present in most mammalian muscles at birth, although more nuclei are incorporated into myofibres during postnatal development. In the event that a muscle fibre is damaged, it undergoes complete degeneration. After the cellular debris is removed by typical phagocytic mechanisms, a completely new muscle fibre will form in its place. These new fibres arise from a stem cell population that consists of small undifferentiated mononucleated cells, known as satellite cells. These are named for their location between the cell membrane and the basal lamina of a normal muscle fibre. On degeneration of a muscle fibre, the satellite cells, which are normally quiescent, are induced to divide, and some of the daughter cells will differentiate into myoblasts. The mononucleated myoblasts subsequently fuse to form polynucleated myotubes. These represent immature muscle fibres. Continued differentiation will result in a fully mature muscle fibre within about 3 weeks[17,29]. Although this process is referred to as 'regeneration', the original muscle fibre does not repair its damaged self, as occurs with neuronal regeneration, but is replaced by a completely new fibre.

Direct gene transfer is about ten times more efficient in regenerating than normal mature muscle, as demonstrated by the level of luciferase activity following injection of pCMV-luc. This is probably due to transfection of a greater number of fibres rather than an increased level of expression per transfected fibre, since approximately ten times more fibres stained for β-galactosidase in regenerating than in normal muscles injected with pCMV-lacZ. The increased transfection is

probably due to more than one factor. For example, there may be improved distribution of the DNA solution in the muscle since the small newly formed muscle fibres may leave more interstitial space. In addition, it is possible that gene transfer may be more efficient in less mature than in the fully mature fibres. Although our results indicate that the regenerating muscle fibre must attain a certain level of maturity before gene transfer can take place. Injection of plasmid DNA as early as 1 or 2 days after degeneration was induced, when mononucleated myogenic cells are present, resulted in no detectable reporter gene expression. At 3 days, when myotubes and young myofibres predominate, the efficiency of transfer was roughly the same as in normal mature muscle[3]. However, the efficiency of transfer increased dramatically by 5 days and remained elevated for up to 11 days post-degeneration. The increased levels of transfection were obtained when the DNA was injected into muscles with newly reformed fibres and this period coincides with maturation of the myotube into a muscle fibre and development of the T-tubule system. Furthermore, the expression of β-galactosidase was observed in the muscle fibres, not the satellite cells. Thus, the mononucleated dividing satellite cells do not seem to be involved in plasmid DNA uptake *in vivo*, a finding which is in agreement with previously reported cell culture studies[31].

DNA-based immunization to HBsAg

Injection of plasmid DNA encoding the S protein of HBsAg into normal or regenerating mouse TA muscle resulted in a high and sustained humoral response[18]. Similarly, immunization in rats using the Biojector® for intramuscular injection resulted in a superior humoral response in regenerating than in normal mature muscle. Both an earlier appearance and higher levels of antibody were observed in regenerating muscle, although all animals eventually became seropositive and attained high levels of antibody. Thus, despite the low efficiency of gene transfer in normal mature muscle, it is still adequate for DNA-based immunization. The superior immune response obtained using regenerating muscle is certainly due, at least in part, to a greater number of muscle fibres taking up and expressing the DNA. However, it is possible that other factors are also involved. For example, regenerating muscle, at least in the early stages, probably contains more mononucleated cells, some of which can act as efficient antigen-presenting cells (e.g. macrophages or dendritic cells). In addition, the level of expression of MHC class I molecules is greater on the surface of immature than mature muscle fibres[32]. Finally, muscle may secrete cytokines which could influence an immune response and these may be different or more abundant in regenerating than in normal muscle tissue.

Use of the Biojector® jet injection system for DNA-based immunization

Needleless jet injection systems might provide an attractive method for administration of plasmid DNA for the purpose of intramuscular immunization. Not only do they provide the benefit of avoiding needlestick injury, but they may produce a better distribution of injected substances in the muscle. Direct gene transfer was first demonstrated on mammary tissue using the Ped-O-Jet system, but this apparatus was designed largely for intradermal or subcutaneous injections[33]. In contrast, the Biojector® jet injection system is capable of intradermal, subcutaneous or intramuscular delivery, depending on the size of the outlet orifice in the syringe. We have shown here that DNA-based immunization to HBsAg in rabbits is superior with the Biojector® than with a classical syringe and needle. This may be due to factors other than simply an improved efficiency of transfection, since luciferase reporter gene expression after injection of pCMV-luc in rat hindlimb muscles was not significantly different when injected by needle or Biojector® (data not shown). In any event, rat muscles similarly injected with a *lacZ*-expressing construct never exhibited more than ten muscle fibres staining positive for β-galactosidase. This underlines the fact that very few muscle fibres need to be transfected for the purpose of DNA-based immunization.

In conclusion, a single intramuscular injection of plasmid DNA encoding a protein antigen is an efficient means of inducing a rapid and sustained humoral response. This is particularly true when the gene transfer is carried out in regenerating muscle. The greater effectiveness in regenerating muscle would be explained not only by increased DNA uptake but also by a more vigorous response due to the overall mechanism by which the antigen is presented to the immune system. Such an approach to immunization may find applications in humans in therapeutic situations. The outcome after injection of DNA into normal mature muscle is not as good as that in regenerating muscle, and clearly further practical improvements will be required to render DNA-based immunization a realistic alternative to classical vaccination. Finally, DNA-based immunization is clearly of great value for basic immunological research and vaccine development, particularly because of the rapidity with which new concepts and vaccine strategies can be tested directly in an *in vivo* setting.

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Exhibit B

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DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibody

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The possibility of inducing an immune response to a protein expressed directly from an introduced gene represents an alternative to classic vaccination. We evaluated the ability of plasmid-based eukaryotic expression vectors to produce the Hepatitis B surface antigen (HBsAg) after injection of pure DNA into mouse tibialis anterior muscles. DNA was injected into either normal mature muscle, or regenerating muscle following cardiotoxin-induced degeneration. The sera obtained from these animals contained significant levels of HBsAg as early as 10 days after gene transfer, at which time low levels of antibodies to HBsAg (anti-HBsAg) were already present. Between 15–60 d after DNA transfer, serum levels of anti-HBsAg steadily increased whereas those for HBsAg fell, most likely due to the neutralizing effect of the antibodies. Analysis of proportions of HBs-seropositive mice showed that within 2 wk of injection of 100 μg pCMV-HBs in regenerating muscle, 91% of the mice were seropositive [defined as having more than 1 milli-International Unit/ml (mIU/ml) of anti-HBsAg]. Even at that early time, 68% had titers of anti-HBsAg greater than 10 mIU/ml, a level that is recognized as being sufficient in humans to confer protection against natural Hepatitis B virus infection. The proportion of seropositive animals rose to 95% by 4 wk, and 100% by 8 wk, at which time all mice had greater than 100 mIU anti-HBsAg in their sera. We have thus demonstrated that direct intramuscular injection of a plasmid vector encoding the HBsAg will give rise to secretion of the viral surface protein into the circulation which leads to an appropriate antibody response. Obtaining secretion of foreign proteins could have several therapeutic uses, particularly in situations where production at low levels is required over long periods of time.

INTRODUCTION

Hepatitis B is a widespread and serious international health problem. There are approximately 300 million chronic carriers worldwide, and while this involves less than 0.5% of the population in Europe and North America, the prevalence rate is 10 to 20% in China, Southeast Asia and subsaharan Africa (1). In addition to causing acute hepatitis and liver damage, the Hepatitis B virus (HBV) can eventually cause cirrhosis and hepatocellular carcinoma. This is particularly likely in the 80–90% of neonatally infected infants and 5–15% of infected adults who become chronically infected carriers (2, 3).

The HBV is a 42-nm particle (Dane particle) consisting of a lipoprotein envelope enclosing a core protein (capsid) and the viral genome, which contains only four genes (S, C, P, X). The major (or small) envelope protein, which includes the Hepatitis B virus (HBsAg), is encoded by the S gene and is organized into dimers of glycosylated and unglycosylated polypeptides (4). Present in smaller amounts are the middle and large envelope proteins, which are encoded by the pre-S2 and S or pre-S1, pre-S2 and S genes respectively. The predominant form of HBsAg secreted by infected cells is not the Dane particle however, but 22-nm particles or filaments which are empty viral envelopes composed solely or predominantly of major (small) envelope protein and sometimes small amounts of middle and large protein (5). These 22-nm particles are seen to persist in the plasma of chronic carriers.

The first vaccine against HBV to be applied to humans (in 1976) involved injection of 22-nm HBsAg particles, which had been purified from the plasma of chronic carriers (6). Although the plasma-derived protein vaccine was effective, it was not appropriate for mass immunization owing to the long and expensive purification procedure, the need to assay each batch on chimpanzees for safety, and most importantly, the limited supply of chronically infected human plasma (7, 8). Currently used are recombinant protein vaccines based on HBsAg-producing yeast cells (9) or Chinese hamster ovary cells (10), which contain the S or the S plus pre-S2 genes respectively. Eukaryotic cell lines are used since they are capable of producing HBsAg particles, which are about 1000 times more immunogenic than unassembled HBsAg protein (11). These recombinant protein vaccines are highly effective and safe but have time-consuming

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and expensive production procedures. Even though viral vaccines are more efficient than protein vaccines, they have not been applied to HBV owing to safety considerations.

A DNA-based vaccine involves the transfer of a gene, by direct or indirect means, such that the protein subsequently produced acts as an antigen and induce a humoral- and/or cellular-mediated immunological response. Genetic immunization against a recombinant protein was first demonstrated (in 1992) in mice, in which the gene for human growth hormone (hGH) was introduced biolistically into epidermal cells in the form of DNA-coated gold particles; the mice subsequently exhibited circulating antibodies against hGH (12). More recently, direct gene transfer of plasmid DNA into skeletal muscle has been used to immunize mice against influenza A (13) and HIV (14).

Gene transfer into skeletal muscle can be carried out directly using intramuscular injection of adenovirus (15, 16), retrovirus (17, 18) or pure plasmid DNA (19–27). Regardless of the method used, gene transfer is more efficient in regenerating than mature muscle; in regenerating muscle adenovirus and plasmid DNA give equivalent results and are both superior to retrovirus, whereas in normal mature muscle pure plasmid DNA is superior to the viral vectors (20). Although it has been shown that naked DNA does not appear to become integrated into genomic DNA, expression of reporter genes persists for up to 19 months (27).

The possibility of inducing an immune response to a protein expressed directly from an introduced gene represents an attractive alternative to classic vaccination, provided that the DNA to be expressed can be introduced effectively and safely. Using intramuscular injection of purified recombinant plasmid DNA, we have introduced expression vectors for the surface antigen of Hepatitis B virus (HBsAg) into normal or regenerating mouse muscle and show that HBsAg is subsequently found in injected muscles and serum. Circulating antibody to the particulate form of HBsAg, which appears as early as 10 days and persists for at least several weeks, reaches levels greatly superior to those which confer protection against infection in humans.

RESULTS

Myogenic C2 cell line secretes recombinant HBsAg

We have cloned expression vectors containing the HBV S gene driven by the CMV or RSV promoter (pCMV-HBs and pRSV-HBs). When transfected into mouse L cells, these vectors produced levels of secreted HBsAg comparable to those transfected with a control HBs expression vector (pSV2S) (28) (Fig. 1, upper panel) We tested these vectors in myogenic cell culture using the mouse C2 cell line to test the ability of the constructs to produce HBsAg in developing muscle. Accumulation of HBsAg increased steadily during the phase of myogenic cell fusion and differentiation, with the two cloned vectors giving equivalent results (Fig. 1, lower panel). This shows that production and secretion of HBsAg is compatible with myogenic differentiation.

Mice produce antibodies to HBsAg particles secreted by muscles injected with plasmid DNA

We then tested the ability of the pRSV-HBs and pCMV-HBs expression vectors to produce protein after intramuscular injection in mice. Normal mature or regenerating (5 days following cardiotoxin-induced degeneration) mouse tibialis anterior (TA) muscles were injected on only one occasion with pCMV-HBs or pRSV-HBs such that each animal received a total of 100 or

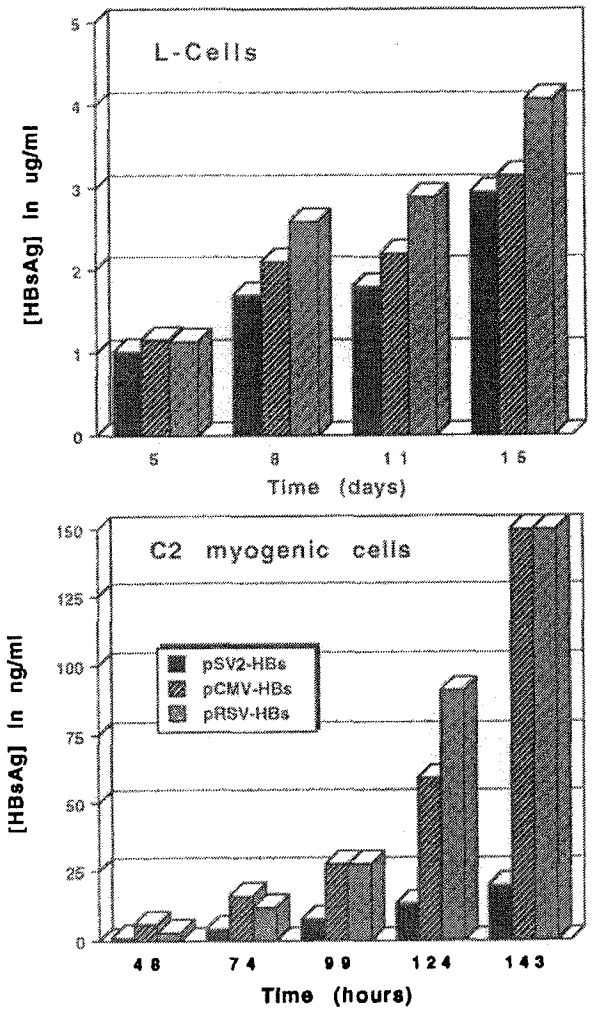

Figure 1. Expression and secretion of HBsAg in transiently transfected cultures of mouse L cells (upper panel) and the mouse C2 myogenic cell line (lower panel). At various times after transfection with the HBsAg expression vectors pRSV-HBs, pCMV-HBs or pSV2S, aliquots of culture supernatant were removed for measurement of secreted HBsAg. The C2 cultures underwent differentiation during the time of the experiment. At 48 hrs after transfection the cells were not yet fused, between 74 and 124 hrs the cells were well fused and the myotubes increased in size, and at 143 hrs, myotubes had begun to detach from the culture dish. Thus, HBsAg was synthesized and secreted during the period of active cell fusion and myotube differentiation.

200 μg DNA; muscles received no further injections of DNA or any other treatment. Sera obtained from these animals contained significant levels of HBsAg as early as 10 days after gene transfer, at which time low levels of antibodies to HBsAg (anti-HBsAg) were already present (not shown). Between 15–60 d after DNA transfer, serum levels of anti-HBsAg steadily increased whereas those for HBsAg fell, most likely due to the neutralizing effect of the antibodies (Fig. 2). Levels of anti-HBsAg rose more quickly in mice injected with 200 rather than 100 μg plasmid DNA (Fig. 2, lower panel).

The mouse immune system is an appropriate and well-studied model to evaluate HBV vaccination, and the findings from it are applicable to humans (29). Analysis of proportions of HBs seropositive mice showed that within 2 wk of injection of 100 μg

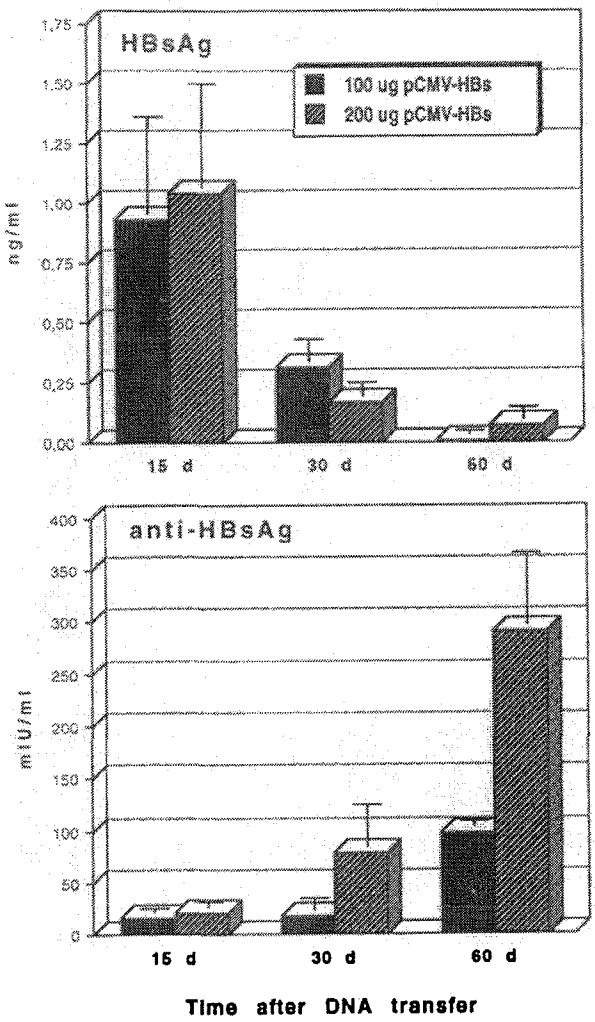

Figure 2. Amounts of HBsAg and anti-HBsAg in sera of mice following direct intramuscular injection of 100 μg or 200 μg (100 μg unilateral or bilateral injections respectively) of pure plasmid DNA encoding HBsAg (pCMV-HBs) into regenerating (5 d after cardiotoxin-induced degeneration) TA muscles. Significant amounts of both HBsAg and anti-HBsAg were 33detected at 15 d (n=6). With longer periods of time (30 and 60 d, n=4) levels of HBsAg decreased while those for anti-HBsAg increased.

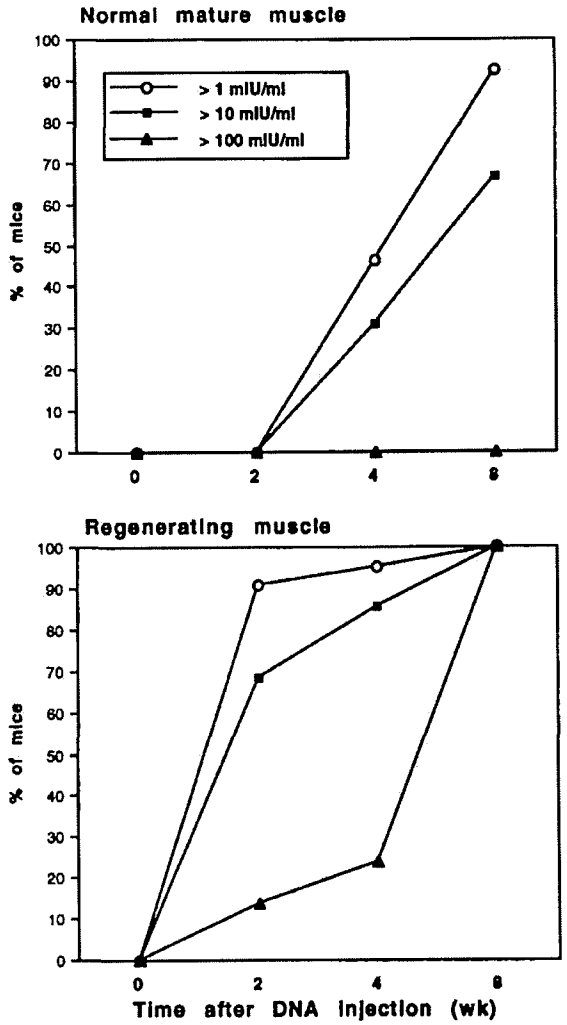

Figure 3. Proportions of mice found to be seropositive for anti-HBsAg at various times after injection of 100 μg of pCMV-HBs (bilateral injections of 50 μg) into normal mature (n=13) or regenerating (5 d after cardiotoxin treatment) TA muscles (n=13). Gene transfer into regenerating muscle resulted in earlier and greater seropositivity than with injection of the DNA into normal muscle.

pCMV-HBs in regenerating muscle, 91% of the mice were seropositive, which we have arbitrarily defined as having more than 1 milli-International Unit/ml (mIU/ml) of anti-HBsAg, since this is within but near the lower detectable limit of the EIA assay procedure used. Even at that early time, 68% had titers of anti-HBsAg greater than 10 mIU/ml, a level that is recognized as being sufficient in humans to confer protection against natural HBV infection (30). The proportion of seropositive animals rose to 95% by 4 wk, and 100% by 8 wk, at which time all mice had greater than 100 mIU anti-HBsAg in their sera (Fig. 3). Similar proportions of seropositive animals were obtained using 100 μg pRSV-HBs in regenerating muscle, although absolute levels of anti-HBsAg in the sera were about half those in animals injected with the same quantity pCMV-HBs (not shown). This is consistent with our finding that the CMV promoter is significantly stronger than the RSV promoter in muscle tissue (H.L.Davis & R.G.Whalen, unpublished results).

In contrast to the rapid development of anti-HBsAg following gene transfer in regenerating muscle, was the slower time-course of antibody appearance after introduction of DNA into normal muscle. Mice in which normal mature muscles were injected with 100 μg pCMV-HBs exhibited no anti-HBsAg in their sera at 2 wk, however 46% converted to seropositivity by 4 wk, and by 8 wk this increased to 92%, with 62% having greater than 10 mIU/ml (Fig. 3). The time course of antibody induction would seem to be a physiological correlate of the 10-fold greater efficiency of gene transfer into regenerating versus normal muscle that we have observed with reporter plasmids (20). While this may argue for further improvements of gene transfer into normal muscle, protective levels of antibodies were still exhibited in less time than it takes to carry out a routine immunization schedule. In addition, it supports the idea that gene expression from the HBsAg expression vector continues for many weeks.

Muscles removed 30 or 60 d after injection of pCMV-HBs contained significant quantities of HBsAg (8.8 ± 1.1 ng/muscle

or 1.1 ± 0.1 ng/mg protein; mean ± SE, n = 14). Immunohistochemical detection of HBsAg particles on cryostat sections of such muscles revealed fibers expressing HBsAg, with homogenous cytoplasmic staining (not shown). The antibodies used to detect HBsAg in the ELISA assay and immunohistochemically on sections are both known to react solely with the particulate form of antigen, as evidenced by failure to detect HBsAg on Western blots.

DISCUSSION

Our results demonstrate that muscle is capable not only of synthesizing a foreign protein, but also of processing and secreting it into the circulation. This is the first clear demonstration of secretion of a foreign protein by muscle tissue after synthesis from a cloned gene introduced by direct transfer of pure recombinant plasmid DNA. Since expression continues for long periods, as is evidenced by continually increasing antibody titres for at least two months, we have concluded that the antigen enters the circulation by secretion rather than release from damaged muscle fibres. These observations concerning HBsAg production and secretion suggest that other proteins not normally produced by skeletal muscle could be metabolized appropriately. Obtaining secretion of foreign proteins could have several therapeutic uses, particularly in situations where secretion at low levels is required over long periods of time.

As expected for the secretion of a heterologous protein, in this case of viral origin, antibodies were produced against the antigen. The humoral-mediated immunity found is appropriate to circulating HBsAg, and it is known that a humoral response alone is sufficient to confer protection against HBV infection in humans. The mouse is a practical and accepted model for calibrating and validating the recombinant protein vaccines against HBV for use in humans (2, 6–11). The apparently constant secretion of the HBsAg creates a novel situation concerning the nature of the response of the immune system. It seems reasonable to assume that the presence of low levels of antigen, over a long period of time provides a favorable scenario for the purpose of immunization. Indeed, in the present work the serum concentration of secreted HBsAg after a one-time injection of DNA, which was probably less than that following an injection of recombinant protein vaccine at the ED_{50} (10), was sufficient to induce the production of anti-HBsAg by the earliest time point examined (10 days after injection) and the antibody levels continued to increase for up to at least 60 days. The recent demonstration of a protective immunological response against influenza A following injection of a plasmid DNA capable of expressing the viral nucleoprotein is thought to result not from secretion, but rather from intracellular processing of the antigen and surface presentation via class I major histocompatibility complex (MHC) molecules (13). The role of a cytotoxic T lymphocyte (CTL) response for HBV is unknown, but even though it may not be necessary for protection in the case of Hepatitis B, a CTL response may be essential in other circumstances. If a CTL response were to occur, one potential consequence would be the eventual destruction of the transfected muscle fibres. This situation would be tolerable since the destroyed fibres would be capable of regeneration, and at least in the case of HBsAg, it would not occur until after a sufficient immune response was produced. Nevertheless, it will be important to characterize the immune response of genetic vaccines, such as the types of immunoglobulins produced and the presence of CTL, since the exact nature of the response may indicate limitations in potential applications or suggest new ones both for basic and applied research.

There are two potential concerns in the use of DNA as the injectable substance for vaccination. First, even plasmids containing no obvious recombinogenic DNA sequences (e.g. viral long terminal repeats) could potentially be incorporated into the cellular genome, with the attendant possibility of insertional mutagenesis. In principle, this event should be much less likely with plasmid DNA than with the genomes of adenoviral and retroviral vectors, which are frequently employed for gene transfer and are currently being tested in human gene therapy trials (31). Moreover, pure DNA seems to be taken up by skeletal muscle fibers far more efficiently than by other cell types. Since the nuclei of muscle fibers are permanently post-mitotic, the frequency of DNA integration as well as the possibility of a mutagenic event leading to cancerous growth is greatly diminished. Nonetheless, such a concern is justified and must be addressed by experimental approaches before mass vaccination programs could be envisioned.

The second concern, in consideration of the finding that gene transfer is less efficient into muscles of primates than of rodents (22), is whether the efficiency of transfer in humans would be sufficient to produce an immunological response. In the case of HBV, vaccination in endemic regions would be carried out on infants within the first week of life, when muscles are still immature and would resemble those found in the regeneration model used in the present study (32). Vaccination of adults might require the use of several injection sites in order to ensure uptake of sufficient DNA, or alternatively, some form of preinjury might be considered. In view of these considerations, and the fact that expression is relatively long-lasting, it will be important to determine the effective dose as a function of time.

Despite these two concerns related to genetic vaccination of humans, there are other situations in which the injection of pure plasmid DNA for therapeutic purposes, with or without preinjury, could have potential benefits that would clearly outweigh the risks involved (e.g. HIV infection and cancer).

In conclusion, we have demonstrated that direct intramuscular injection of a plasmid vector encoding the HBsAg will leads to to secretion of the viral surface protein into the circulation, apparently in the form of empty particles. The resulting immune response produces antibody levels in mice that are greater than those obtained after injection of naturally occurring or recombinant empty viral envelopes (10). The speed and efficacy of the production of antibody suggests the use of this methodology for the purpose of vaccination. The use of pure recombinant DNA as the injected substance offers many technical, economical and logistical advantages including the ease and low cost of production and ease of quality control compared with protein- or virus-based vaccines. The induction of both humoral- and cellular-mediated immune responses should make the efficacy of DNA-based vaccines equal to that of viral vaccines, but without the important attendant risk factors. In addition, the unique feature of longevity of gene expression and the continuous presentation of the antigen at low levels to the immune system obviates the need for booster injections, which is of importance considering that the greatest need for mass vaccination is in developing regions of the world, where it is often difficult to recontact individuals after an initial vaccination, especially when migratory populations are involved. Another important advantage of such vaccines is the ease by which the DNA sequences to be expressed could be varied to

respond to different or changing strains of pathogens. Furthermore, the demonstration that skeletal muscle tissue is capable of secreting a relatively complex non-muscle protein expressed from injected recombinant DNA indicates that other types of therapeutic applications should be possible with this methodology.

MATERIALS AND METHODS

Expression vectors

The plasmid pCP10, which contains two copies of the HBV genome head-to-tail (10, 33), was the source of the Hepatitis B surface antigen gene. A *XhoI*−*BglII* restriction fragment containing the S gene, the necessary signal sequences for secretion and downstream untranslated sequences including the polyadenylation signal, was prepared from this plasmid and cloned into the corresponding sites of a Bluescript vector, which had been modified to contain extra cloning sites in the polylinker (provided by Dr S.Tajbakhsh). The S gene-containing fragment was then removed by *KpnI*−*BssHII* digestion, and this fragment was cloned into the corresponding sites of the expression vectors pRc/RSV and pRc/CMV (Invitrogen). The final constructs were designated pRSV-HBs and pCMV-HBs, respectively. The HBs expression vector pSV2S has been described previously (28).

The plasmid DNA used in all experiments was prepared using Qiagen DNA purification columns (Diagen, Hilden, Germany).

Ex vivo gene transfer

Transfection was carried out with L cells using the DEAE-dextran method (34), and with C2 cells using the lipid compound DOGS, as previously described (35).

In vivo gene transfer

The HBsAg expression vectors were injected directly into normal and regenerating (5 days after cardiotoxin-induced degeneration) tibialis anterior (TA) muscles of male C57BL/6J mice weighing 19−21 g (approximately 6−7 wk of age). Plasmid DNA was injected as previously described (21), such that each animal received either 100 μg (100 μg unilaterally or 50 μg bilaterally) or 200 μg (100 μg bilaterally) of pCMV-HBs or pRSV-HBs in PBS. Each muscle was injected with a volume of 50 μl (1 or 2 μg DNA/μl PBS). Normal mature TA muscles were pretreated (30 min prior to gene transfer) by injection with 50 μl hypertonic sucrose (25% w/v in PBS), since this has previously been shown to reduce the variability of efficiency of direct gene transfer into skeletal muscle (21). Regenerating TA muscle was produced by inducing a single cycle of myofiber degeneration and regeneration by intramuscular injection of cardiotoxin (50 μl of 10 μM in PBS), which was purified from the venom of the *Naja nigricollis* snake (Latoxan, Rosans, France), using a 27g needle fitted with a collar of PE tubing which limited penetration to 2 mm. All intramuscular injections of cardiotoxin, sucrose solution or plasmid DNA were carried out under anesthesia (sodium pentobarbital, 75 mg/kg IP).

Collection and preparation of sera and muscles from mice

At various times following gene transfer, blood was collected from anesthetized mice by retrobulbar puncture using heparinized glass pipettes and serum, which was recovered by centrifugation (10 min at 8000 g), was stored at −20°C until used for assay of HBsAg or anti-HBsAg. At the termination of the study, animals were killed by cervical dislocation and entire TA muscles were removed, pulverized in liquid nitrogen and homogenized in 500 μl lysis buffer (25 mM Tris, pH 7.4, 1 mM $MgCl_2$, 5 mM KCl) for 1 min with Kontes plastic pestle. The homogenate was allowed to extract on ice 1 min, then after the addition of 50 μl 0.1% NP40, was incubated for an additional 15 min on ice then centrifuged for 5 min at 8000 rpm. The supernatant was stored at −20°C until used for assay of HBsAg.

Assay of HBsAg and anti-HBsAg

The amount of HBsAg in cell culture supernatants and muscle extracts was measured using a commercial ELISA method (Monolisa AgHBs, Diagnostics Pasteur, Paris). Amounts of HBsAg and anti-HBsAg in mouse serum were determined using commercial monoclonal enzyme immunoassay methods (AUSZYME and AUSAB respectively, Abbott). HBsAg was detected on cryostat sections (not shown) following brief fixation in cold ethanol using rabbit serum to HBsAg as primary antibody and donkey anti-rabbit IgG-Texas red (Amersham, France) as secondary antibody.

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Exhibit C

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Immunology

DNA-mediated immunization to the hepatitis B surface antigen in mice: Aspects of the humoral response mimic hepatitis B viral infection in humans

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ABSTRACT **Intramuscular injection of plasmid DNA expression vectors encoding the three envelope proteins of the hepatitis B virus (HBV) induced humoral responses in C57BL/6 mice specific to several antigenic determinants of the viral envelope. The first antibodies appeared within 1–2 weeks after injection of DNA and included antibodies of the IgM isotype. Over the next few weeks, an IgM to IgG class switch occurred, indicating helper T-lymphocyte activity. Peak IgG titers were reached by 4–8 weeks after a single DNA injection and were maintained for at least 6 months without further DNA injections. The antibodies to the envelope proteins reacted with group- and subtype-specific antigenic determinants of the HBV surface antigen (HBsAg). Expression vectors encoding the major (S) and middle (preS2 plus S) envelope proteins induced antibodies specific to the S protein and the preS2 domain, and preS2 antibodies were prominent at early time points. In general, the expression vectors induced humoral responses in mice that mimic those observed in humans during the course of natural HBV infection.**

DNA-mediated immunization refers to the induction of an immune response to antigen expressed *in vivo* subsequent to the introduction of DNA carrying the protein coding sequences and the regulatory elements needed to express them (for review, see ref. 1). An important feature of DNA-based immunization is the *in situ* production of the expressed protein(s), mimicking in this respect a viral infection. This endogenous synthesis should allow presentation of antigens by class I molecules of the major histocompatibility complex (MHC) and thus result in the induction of $CD8^+$ cytotoxic T lymphocytes (CTL) (2). Therefore, the potential of DNA-mediated immunization to partially mimic viral infection promises the efficacy of live attenuated vaccines without the risk of inadvertent infection (3). Another attractive and important feature is the ease of designing expression vectors including sequences chosen to induce a desired immune response. There have been several reports of animal models in which pure recombinant plasmid DNA was used to induce immune responses to proteins of pathogens, including influenza A (4–9), human immunodeficiency virus type 1 (10, 11), bovine herpes virus (12), rabies virus (13), malarial parasites (14), and, in our own earlier work, hepatitis B virus (HBV) (15, 16). In these studies, DNA-based immunization was shown to induce a broad range of immune responses, including neutralizing antibodies, CTL, T-cell proliferation, and (where evaluation was possible) protection against challenge with the pathogen.

The structural gene encoding the HBV envelope proteins carrying the surface antigen determinants (HBsAg) has a single open reading frame containing three in-frame ATG start codons that divide the gene into three coding regions known as preS1, preS2, and S (proceeding in a 5′ → 3′ direction) (see refs. 17 and 18 and Fig. 1). The three different-sized polypeptides produced are known as the major (S), middle (preS2 plus S), and large (preS1 plus preS2 plus S) envelope proteins, and these spontaneously assemble into subviral particles. Protection against HBV infection in humans can be achieved by inducing antibodies to the viral surface antigenic determinants.

In this paper we compare the fine specificity of the humoral response obtained after DNA-mediated immunization using several plasmid vectors encoding the different HBV envelope proteins. We find that the humoral immune response induced by the *in situ* production of the protein antigen(s) in mice can mimic aspects of that which occurs during natural infection in humans.

MATERIALS AND METHODS

HBV Envelope Plasmid Expression Vectors. The plasmid pCP10 (19) was the source of the envelope coding sequences and the 3′ untranslated sequences that include the viral poly(A) signal. In three of the plasmids (pCMV-S, pCMV-S1.S2.S, and pCMV-S2.S) the HBV sequences were placed under the transcriptional control of the human cytomegalovirus (CMV) immediate early promoter, whereas the fourth construct (pHBV-S2.S) used the endogenous HBV promoter elements situated in the preS1 protein coding region of the envelope gene (see Fig. 1).

Plasmid pCMV-S. This construct, which has been described previously (15), expresses only the S region of the HBV envelope gene.

Plasmid pHBV-S2.S. A 2.4-kb *Bgl* II–*Bgl* II restriction fragment from pCP10 (containing the preS1, preS2, and S coding sequences) was cloned into the *Bam*HI site of a modified pSK Bluescript vector. The endogenous HBV promoter within the preS1 region drives expression of the major and middle HBV envelope proteins.

Plasmid pCMV-S1.S2.S. A 2.6-kb fragment was removed from the pHBV-S2.S vector using the Bluescript *Bgl* II and *Bss*HII sites and cloned between the *Bam*HI site of the polylinker of pcDNA3 (Invitrogen) and the *Bss*HII site within the neomycin gene of the vector. This generated plasmid pCMV-S1.S2.S.

Plasmid pCMV-S2.S. The pCMV-S2.S vector was generated by digesting pCMV-S1.S2.S with *Kpn* I and *Sau* I, treated with

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Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; mIU, milli-international units; CMV, cytomegalovirus; MHC, major histocompatibility complex.

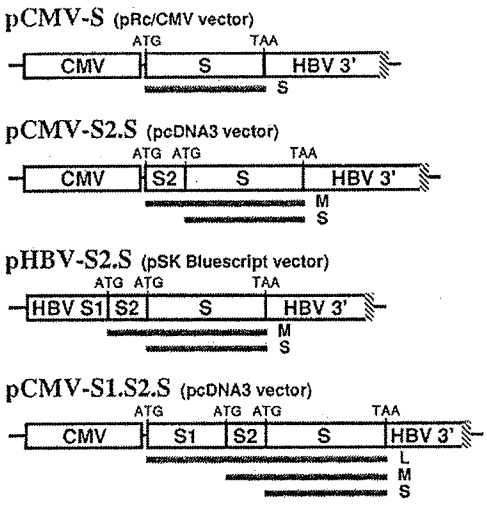

FIG. 1. Schematic diagram of the HBV envelope expression vectors. The preS1, preS2, and S sequences are indicated along with the three in-frame ATG initiator codons and the TAA stop codon. The expected protein products are indicated as thick gray lines below the respective coding sequences and represent the major surface protein (S) as well as the middle (M) and large (L) envelope proteins.

S1 nuclease, and ligated, thus deleting most of the preS1 sequences.

The plasmid DNA used for cell culture transfection and *in vivo* injection was prepared using Qiagen DNA purification columns (Diagen, Hilden, Germany) as described (15).

***In Vitro* Transfection with HBV Envelope Expression Vectors.** *Transfection of cell lines.* Mouse L cells and C2-18 mouse myoblasts were transiently transfected as described (20, 21). At various times after transfection, aliquots of culture supernatants were removed for determination of the amount of HBsAg particles secreted.

Detection of HBV envelope proteins by ELISA. The HBsAg particles secreted from transfected cells or those that remained intracellular (recovered by three rounds of freezing and thawing of the harvested cells) were quantitated using a commercial ELISA kit (Monolisa AgHBs, Diagnostics Pasteur, Marnes la Coquette, France). The proportion of the different envelope proteins in particles produced from the various plasmids was determined with a specific sandwich ELISA assay using different monoclonal antibodies (mAbs) bound to the solid phase. These included a group-specific anti-S (112A26 from J. P. Bourgeois, Diagnostics Pasteur), an anti-preS2 (F-124 from A. Budkowska, Pasteur Institute; ref. 22), and an anti-preS1 (MA18/7 from W. Gerlich, University of Göttingen, Göttingen, Germany). Peroxidase-labeled anti-S mAbs 6-16-A15 and 144A2 (also from J. P. Bourgeois), which do not react with the previous three, were used as probes. Supernatant recovered from cultures of cells transfected with pSVS plasmid (23) was used as a positive control for the presence of the preS1 and preS2 epitopes.

DNA-Based Immunization of Mice. In vivo *gene transfer.* Each expression vector was injected into 5-day regenerating tibialis anterior muscles of groups of eight 6- to 8-week-old (19–21 g) male mice of the C57BL/6 strain ($H\text{-}2^b$) as described (24).

Measurement of in vivo *antibody production.* At various times following gene transfer, pooled sera were tested for reactivity against a panel of antigens. Since group and subtype determinants of HBsAg are conformational (25), whole particles rather than peptides were used as the solid phase for the ELISA. Particles of a different (*adw*) or same (*ayw*) subtype were used to determine group (*a*) and group plus subtype (*y*) specific titers, respectively (26). The preS2-specific response was determined as the difference between results obtained using particles with and without the preS2 domain. The preS2 domain had been removed from the middle protein of Chinese hamster ovary-derived particles (23) by treatment with trypsin (1 μg/ml) as described (27). Antibodies to the preS regions of the HBV envelope were also quantitated using synthetic peptides corresponding to amino acid sequence 12–49 (preS1) or 120–145 (preS2) of the *ayw* subtype or sequence 94–117 (preS1) of the *adw* subtype.

RESULTS

Expression of HBV Envelope Proteins Following Transfection of Cells in Culture. Particles composed of HBV envelope proteins were synthesized and secreted from mouse L cells or differentiated C2 myotubes transiently transfected with each of the expression vectors (Fig. 2 *Upper*). With L cells, the greatest accumulation of particles in the culture supernatant was obtained with the pHBV-S2.S vector and the least was with pCMV-S1.S2.S (5000 ng/ml and 40 ng/ml, respectively, at day 14). With C2 myotube cultures, pCMV-S gave the greatest secretion, whereas pCMV-S1.S2.S and pCMV-S2.S gave similar results, although none of the secreted particles contained the large protein (see below).

For those vectors encoding the preS domains, it was important to verify the presence of the expected HBV envelope proteins in the resulting particles (see Fig. 1). Analysis by solid-phase sandwich ELISA confirmed that the preS2 sequence was present in particles secreted by L or C2 cells transfected with pCMV-S2.S, pHBV-S2.S, or pCMV-S1.S2.S plasmid, although each of these produced a different relative proportion of preS2 antigen (Fig. 2 *Lower*). Interestingly, preS1 determinants were detected on particles secreted by L cells but not C2 cells transfected with pCMV-S1.S2.S DNA, even though particles extracted from C2 cells did contain preS1 antigen (results not shown).

Humoral Response to HBV Envelope Proteins in Mice Immunized with DNA Expression Vectors. *Response to the major (S) envelope protein.* Antibodies to HBsAg, which were first detected 1 week after injection of the pCMV-S expression vector, increased to peak ELISA antibody titers of $>10^4$ by 4 weeks and high levels were maintained for at least 6 months despite the absence of further DNA injections (Fig. 3*A*). HBsAg-specific IgM predominated at 1 week after DNA injection, but a class shift to IgG isotypes was observed over the following 2 weeks. The humoral response to the group-specific epitopes clearly preceded that directed to the subtype-specific determinants using this expression vector (compare titers with *ad* and *ay* particles, Fig. 3*A*).

Response to the middle (M) envelope protein. Inclusion of preS2 coding sequences (pCMV-S2.S) resulted in a much stronger early antibody response. At 1 week ELISA, titers for IgM and IgG responses were about 10-fold greater than that seen with pCMV-S (Fig. 3*B*). At this time IgM antibodies predominated and these were almost exclusively preS2-specific. Antibodies to the S domain (group- and subtype-specific) were not detected until 2 weeks after DNA injection. They did not peak until 8 weeks and the peak level was only half that obtained with pCMV-S.

Response to the large (L) envelope protein. With inclusion of preS1 coding sequences (pCMV-S1.S2.S), antibodies appeared about 1 week later than with the other two CMV-based expression vectors (Fig. 3*C*) and, although peak levels were not attained until 12 weeks, they were nevertheless comparable to those obtained with pCMV-S. As with the pCMV-S2.S vector, anti-preS2 immunoglobulins predominated at early times. Antibodies to HBsAg were directed to the group- and subtype-specific determinants.

Response with the endogenous HBV promoter. The pHBV-S2.S vector containing the endogenous HBV promoter expressed the major and middle envelope proteins and resulted in the slowest but strongest humoral response (Fig. 3*D*).

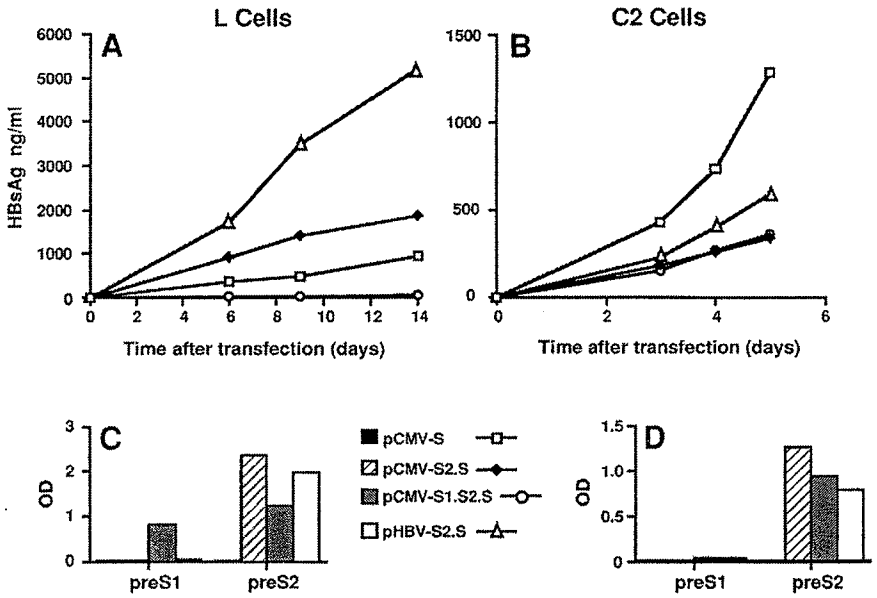

FIG. 2. Expression of HBsAg after transient transfection in cell culture. (*Upper*) The secretion of HBsAg was measured in cultures of mouse L cells (*A*) or the mouse C2 myogenic cell line (*B*) transfected with the different HBV envelope-expressing plasmids. The C2 cells underwent fusion within 2 days after transfection and thus myotubes were present at the time the supernatant was sampled. (*Lower*) The relative amounts of preS2 and preS1 sequences in the HBsAg particles secreted from transiently transfected L cells (*C*) and C2 cells (*D*) were determined. HBsAg particles at a concentration of 25 ng/ml were analyzed for preS2 and preS1 antigenicity by a solid-phase sandwich ELISA method. The results are expressed as OD_{492} units after correction for background.

Antibodies were first detected at 3 weeks and appeared to be directed almost solely against the HBsAg carried by the major protein since similar titers were obtained whether the sera were allowed to react against particles with or without preS2 determinants. However, antibodies to preS2 sequences could be found using synthetic peptides (see below). Maximum anti-HBsAg titers were more than twice as high as those obtained with pCMV-S, and they attained nearly 10^5.

Presence of antibodies to preS regions. Synthetic peptides were used to directly measure the relative proportions of preS2- or

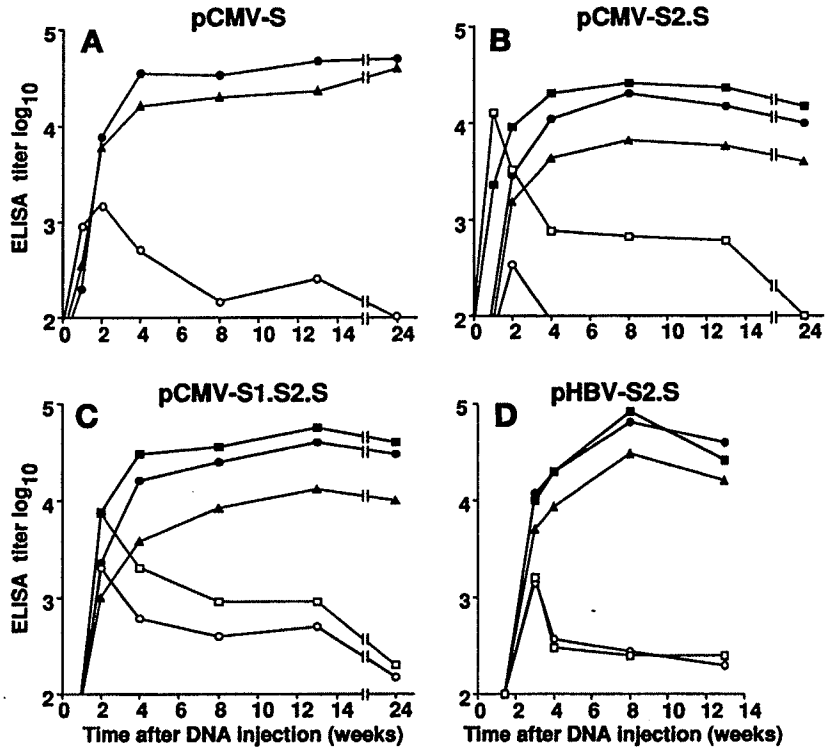

FIG. 3. Kinetics of IgM and IgG anti-HBs antibodies in mice immunized with different HBV envelope-expressing plasmids. Sera were taken at different times after a single DNA injection and pools were made from all sera ($n = 8$) taken at a single time point. The fine specificity of the antibodies was determined using S-containing HBsAg of a homologous (*ay*, ○, ●) or heterologous (*ad*, ▲) subtype as well as HBsAg containing the middle (preS2 plus S, □, ■) protein of the *ay* subtype. The bound antibodies were detected in the second step by the addition of peroxidase-labeled goat anti-mouse IgG (closed symbols) or anti-mouse IgM (open symbols). End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of nonimmune serum (or of pooled sera from mice immunized with an irrelevant DNA) with a cutoff value of 0.050.

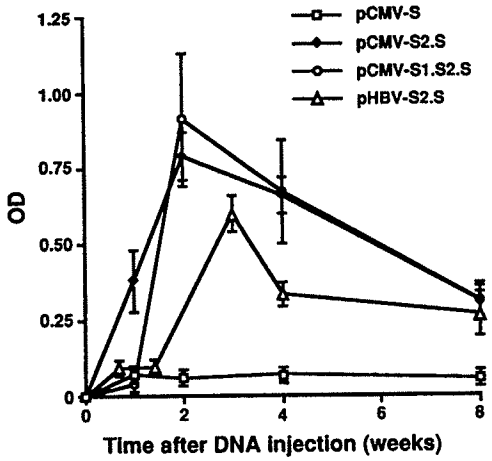

FIG. 4. Kinetics of appearance of anti-preS2 antibodies in sera from groups of mice (n = 8) injected with different HBV envelope-expressing plasmids. Sera were used individually at a 1:100 dilution and the binding to a synthetic preS2 peptide was measured. Bound antibodies were revealed by peroxidase-labeled goat anti-mouse immunoglobulins. Results are expressed as mean ± SEM of the OD at 492 nm.

preS1-specific antibodies induced by the different HBV envelope expression vectors. Antibodies to amino acids 120–145 were detected in the serum of pCMV-S2.S-immunized mice as early as 1 week after DNA injection (Fig. 4). The level of antibodies peaked at 2 weeks and then declined steadily over the next 2 months. In mice immunized with pCMV-S1.S2.S and pHBV-S2.S, antibodies to the preS2 peptide were not detected until 2–3 weeks after DNA injection. Despite their later appearance, they reached levels similar to those which appeared earlier with the pCMV-S2.S vector.

No anti-preS1-specific antibodies were detected using peptides in mouse serum at any time after injection of any of the vectors (results not shown). This is consistent with the cell culture data because, although L and C2 cells transfected with pCMV-S1.S2.S synthesized particles with preS1 polypeptides, only the L cells actually secreted particles with preS1 determinants.

Correlation with clinically defined antibody levels. The antibody levels attained in individual mice were also expressed in milli-international units per ml (mIU/ml) (see Table 1). One week after DNA injection, all mice injected with pCMV-S had seroconverted to a titer of at least 10 mIU/ml [a level that is recognized to be protective against infection in humans (41)] and all those injected with pCMV-S2.S had >100 mIU/ml, although this was largely preS2-specific (compare Fig. 3*B*). In contrast, most mice injected with pCMV-S1.S2.S or pHBV-S2.S failed to seroconvert until after 2 weeks, but by 3 months high levels of antibody had been induced.

DISCUSSION

We have evaluated the humoral immune response obtained after DNA-mediated immunization using four different plasmid DNA vectors for the *in vivo* expression of various HBV envelope proteins. These studies were carried out in mice of the C57BL/6 strain, which are of a haplotype ($H\text{-}2^b$) known to respond well to S and preS2 components of the viral envelope (28). The results presented here show that after a single intramuscular injection of DNA, all vectors induced a strong and sustained antibody response that resembles that of natural HBV infection in terms of the fine specificity, immunoglobulin class, and relative kinetics of the antibodies induced. The group- and subtype-specific determinants are conformational (25) and yet are also well recognized by the antibodies generated after intramuscular injection of DNA expression vectors. These data represent evidence that the envelope proteins encoded by the plasmid DNA have adopted a conformation similar to that of the proteins present during natural infection. This conclusion, drawn from serological evidence, is important because it validates the use of DNA-based *in vivo* synthesis of the antigen for immunization purposes.

All four vectors produce high levels of antibodies specific for group and subtype determinants of the S domain such as appear during resolution of natural infection (29). Antibody levels reach ELISA titers of 10^4–10^5 by 3 months after a single DNA injection and persist at those levels for at least 6 months without further DNA injections. The three plasmid vectors that include preS2 coding sequences induce preS2-specific antibodies, although to different levels, and these antibodies generally appeared earlier than those against the S domain of the envelope proteins. This is similar to the course of the immune response seen in early phases of HBV infection, which is characterized by an immunodominance of the preS2 over the S region (30). The humoral response to preS2 is particularly strong and precocious in C57BL/6 mice when using the pCMV-S2.S vector.

The IgM to IgG class shift, which is also typical of natural infection, indicates that specific helper T cells are probably involved. The T-cell-dependent nature of the humoral response to HBV envelope proteins is well established (see ref. 31); however, neither the phenotype nor the location of the antigen-presenting cells (APCs) that may be involved in T-cell stimulation after DNA injection has been determined. Helper T-cell function is generally an indication of antigen presentation by MHC class II molecules but it is unlikely that this takes place on muscle cells, which do not normally express class II (32). Professional APCs of the leukocyte lineage, such as interstitial dendritic cells in the muscle tissue, could efficiently present the very small quantities of antigen produced (on the order of nanograms; see ref. 15). Thus, with the DNA approach, the kinetics of antigen secretion combined with the residual inflammation from the intramuscular injection could provide favorable conditions for antigen uptake by the dendritic cells and transport to lymph nodes (33, 34). Other cell types might play a role in class II antigen presentation, including macrophages and lymphocytes as well as non-leukocytes such as myoblasts, which have also been shown to express MHC class II (35). Myoblasts are quiescent mononuclear cells found in small numbers in mature muscle and they

Table 1. Anti-HBV immune response in mice injected with the different expression vectors

Vector	Time of 100% seroconversion	Mean titer, mIU/ml		
		1 week	3 months	6 months
pCMV-S	1 week	48 ± 11	937 ± 337	1022 ± 398
pCMV-S2.S	1 week	439 ± 80	466 ± 307	267 ± 168
pCMV-S1.S2.S	2 weeks	1 ± 1	517 ± 188	461 ± 274
pHBV-S2.S	20 days	0 and 3 ± 3*	1725 ± 572	ND

Anti-HBs antibodies were quantitated using a commercial kit (Monolisa anti-HBs, Diagnostics Pasteur, Marnes la Coquette, France) and values were determined relative to the standard provided. Mean titers are expressed in mIU/ml. The threshold for seroconversion was defined as ≥10 mIU/ml. The data are presented as mean ± SEM (n = 8). ND, not determined.

*The two values for the pHBV-S2.S vector were determined at 5 and 10 days, respectively.

proliferate in response to muscle fiber injury such as occurs with the cardiotoxin pretreatment and intramuscular injection used here. Even though muscle fibers are reformed by the time the DNA is injected (36), residual proliferating myoblasts might facilitate the immune response.

It is known from human and primate vaccination studies that antibodies to HBsAg are alone sufficient to confer protection against viral infection. In this regard, a single injection of DNA is able to induce high levels of antibodies to HBsAg in mice that are sustained for at least 6 months. If a comparable response can be attained with DNA in humans, this would offer clear advantages over the current protein vaccines, which usually involve a series of three or four injections given over a 6- to 12-month period. The rapid and strong antibody response to the preS2 region is important for vaccine design since inclusion of the preS2 domain may be beneficial. During natural HBV infection, the presence of preS2 antibodies is a marker of virus clearance from the liver, whereas they are absent in people who progress to a chronic carrier state (37, 38). The preS2 epitopes alone can induce a protective immune response since vaccination of chimpanzees with a synthetic preS2 peptide provides immunity against challenge with HBV (39, 40). Finally, the very rapid appearance of antibodies achieved using the DNA-based immunization may prove particularly beneficial for vaccination against perinatal transmission of the virus, such as occurs in areas where HBV infection is endemic.

DNA-mediated immunization may therefore allow rational design of DNA expression vectors to induce a particular type of immune response. Since changes in the antigen composition and coding sequences can be made and evaluated more rapidly than for recombinant proteins, this approach could also lead to the development of new generations of vaccines based either on plasmid DNA or on novel recombinant proteins.

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